Therapeutic-like Effects of Trace Amine-associated Receptor 1 (TAAR1) Activation in Models of Alcohol Abuse

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Statement of Sources

I declare that this report is my own original work and that contributions of others have been duly acknowledged.

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List of Acronyms

AUD Alcohol use disorder

CNS Central nervous system

DA Dopamine

DAT Dopamine transporter

DSM-V Diagnostic and Statistical Manual of Disorders

I.P Intraperitoneal injection

KO Gene knockout

METH Methamphetamine

MSP Medical Science Precinct

NSMHW National Survey of Mental Health and Wellbeing

SEM Standard error of the mean

TA Trace amine

TAAR1 Trace amine-associated receptor 1

UTAS University of Tasmania

VTA Ventral tegmental area

WT Wild type

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Abstract

Alcohol use disorders (AUD) are one of the most common and devastating forms of drug addiction in Australia. However, current treatments are severely lacking in effective pharmacological options. In recent years, the trace-amine associated receptor 1 (TAAR1) has been identified as a potential target for new pharmacological interventions in drug addiction, showing effectiveness in reducing cocaine- and methamphetamine-addiction related behaviours in animal models. However, the effects of TAAR1 activation on alcohol addiction have not been addressed. This study aimed to examine the potential therapeutic effects of TAAR1 activation with the partial agonist, RO5263397, on alcohol self-administration and locomotor sensitisation behaviours in a rodent model of alcohol addiction. The data showed that TAAR1-activation resulted in a significant decrease of ethanol consumption in g/kg in the first hour of the self-administration test, and a significant decrease in locomotor activity. However, there was no effect on ethanol consumption in mL or preference. Furthermore, TAAR1 activation was also found to significantly reduce sucrose and water consumption (mL) which was unexpected. These findings provide an important first look at the effects of TAAR1 activation on alcohol addiction and provide a strong direction for future studies in this area.

Therapeutic-like Effects of Trace Amine Associated Receptor 1 (TAAR1) Activation in Models of Alcohol Abuse

Alcohol Use Disorder in Australia

Alcohol use disorder (AUD) is a harmful pattern of alcohol use characterised by a number of symptoms, including an excessive and uncontrollable intake of alcohol despite awareness of its negative effects (American Psychiatric Association, 2013). It is graded on a spectrum of severity, which is determined by the number of symptoms present: 2-3 is mild, 4-5 is moderate, and 6 or more is severe and considered to meet the criteria for alcohol abuse (American Psychiatric Association, 2013; Campbell, Lawrence, & Perry, 2018; Freyer, Morley, & Haber, 2016). Alcohol addiction is one of the most persistent and harmful forms of substance addiction, being one of the three biggest contributors to global disease burden (Gowing et al., 2015; World Health Organisation, 2018). An estimated 50% of all alcohol-related harms occur in patients diagnosed with AUD, highlighting the severity of the problem, and making the development of more effective treatments a priority (Gowing et al., 2015; World Health Organisation, 2018).

AUDs are highly prevalent in Australia (Teeson et al., 2010). The 2007 National Survey of Mental Health and Wellbeing (NSMHW) found that 22.1% of the population experienced some form of AUD over their lifetime (Teeson et al., 2010) with AUDs being significantly more common in males and young adults, a finding reflected in international patterns (World Health Organisation, 2018). Lifetime prevalence rates for AUDs in Australia widely vary, with estimates ranging from 4.5% to 17.8%, with it being most common among unmarried, English-speaking males (Teeson et al., 2010). The mean age of onset is approximately 20-22 years, with the hazard rate peaking at 18 (Teeson et al., 2010). Severity of symptoms

tend to escalate throughout the mid to late 20's, which is the age range most people without an AUD decrease in problematic alcohol use behaviours (Freyer et al., 2016).

Over 200 conditions have been linked to the misuse of alcohol, with examples including liver and cardiovascular disease, injuries resulting from road accidents, suicide, tuberculosis, and HIV/AIDS (Freyer et al., 2016; World Health Organisation, 2018). Within Australia, alcohol addiction contributed 2.6% of total disease burden in 2010 (Freyer et al., 2016). What makes alcohol addiction more insidious is that these clinical effects can appear to be blunted and therefore harder to detect in those who are alcohol-dependent, further compounding the risk of negative effects (Freyer et al., 2016). There is evidence that AUD rates are rising in Australia, even as general alcohol consumption decreases, as indicated by an increased rate of hospital admissions for this disorder and other associated conditions such as liver disease (Freyer et al., 2016).

Pharmacological Treatments for Alcohol Use Disorder: The Current State

Due to these detrimental effects, the low rate of success in treating alcohol use disorders is a significant public health concern (Freyer et al., 2016; Revel, Hoener, Renau-Piqueras & Canales, 2012; Teesson et al., 2010). There are numerous factors behind the low success rate, chief of these being the high rate of comorbidity with other substance use disorders, anxiety, and depression, as well as the social stigma attached to the disorder (Campbell, Lawrence & Perry, 2018; Freyer et al., 2016). AUDs are traditionally treated with a combination of psychosocial therapy and pharmacological treatments however the goal of these programs is usually to reduce drinking down to a manageable level and to prevent relapse, rather than

function as an outright cure (Campbell et al., 2018; Freyer et al., 2016; Revel et al., 2012). In situations where the disorder is comorbid with other mental illnesses, AUD is often prioritised in treatment due to its exacerbating effects on other mental illnesses (Freyer et al., 2016; Teeson et al., 2010). In addition to this, numerous public health programs have been implemented around the world in order to reduce the harmful effects of alcohol consumption through education and spreading awareness in an effort to combat some of the social stigma (Campbell et al., 2018; World Health Organisation, 2018). However, these have been largely ineffective in reducing rates of alcohol misuse (Campbell et al., 2018; World Health Organisation, 2018), therefore making the development of new and effective treatments a priority.

In most cases of AUD, the initial withdrawal period after ceasing alcohol consumption is often cited as the most difficult aspect of quitting or controlling intake in patients. These effects are most severe in heavy alcohol drinkers who experience a sudden and substantial decrease in the amount of alcohol they consume (Freyer et al., 2016). Symptoms of alcohol withdrawal can be as minor as tremors, insomnia, headaches, and anxiety, to as severe as seizures and hallucinations, which can require hospitalisation (Freyer et al., 2016). In patients with comorbid disorders, withdrawal symptoms are often exacerbated, along with the symptoms of their comorbid disorders, which can be a very painful and traumatic experience for the patient. It is these severe and complex cases of alcohol abuse that may benefit the most from effective and targeted pharmacological treatments (Freyer et al., 2016; Lynch et al., 2013). However, there are currently no specific drugs for treating either alcohol withdrawal or AUD. Health care professionals and hospitals generally use benzodiazepines such as diazepam, as a short-term treatment (Campbell et al., 2018; Freyer et al., 2016). However, this is ineffective as an ongoing treatment. For long-

term cases, there are currently only three medications approved for this use in Australia: acamprosate, naltrexone, and disulfiram. Acamprosate and naltrexone specifically target the craving symptoms and have been shown to be effective in maintaining abstinence and decreasing alcohol intake in relapsed patients (Campbell et al., 2018; Freyer et al., 2016; Lynch et al., 2013). However, adherence rates remain low and side-effects include diarrhea, fatigue, and nausea (Freyer et al., 2016). Furthermore, acamprosate cannot be taken by patients who have advanced cirrhosis, and/or moderate to severe renal impairment. Disulfiram is different from the first two as it is an active deterrent. Upon consuming or being exposed to alcohol, disulfiram triggers the accumulation of toxic acetaldehyde, causing feelings of nausea (Campbell et al., 2018; Freyer et al., 2016; Lynch et al., 2013). Moreover, this drug can have severe side-effects in some patients which can be fatal, therefore this medication is not recommended unless taken under close professional supervision (Freyer et al., 2016). Finally, all current drugs used to treat alcohol abuse are only effective if the patient has already abstained from alcohol use (Campbell et al., 2018; Lynch et al., 2013). Therefore, there is a need for medications that can target withdrawal symptoms and encourage abstinence when the patient is still consuming alcohol. In doing so, other courses of treatment such as psychotherapy can be maintained, leading to an overall increase in therapy effectiveness and recovery success rates (Revel, Mayer et al., 2012).

The role of the trace amine associated receptor 1 (TAAR1)

Trace amines (TAs) are a family of endogenous molecules with strong similarities to classical monoamines that are now considered a class of neurotransmitters in their own right (Pei et al., 2014). They are present in very low,

trace concentrations in the central nervous system (CNS), several hundred times weaker than classical neurotransmitters, hence their name of 'trace' amine. They are most highly concentrated in the nigrostriatal and mesolimbic dopaminergic pathways such as the ventral tegmental area (VTA), substantia nigra and nucleus accumbens (Pei, Asif-Malik & Canales, 2016). Until recently, they were classified as false neurotransmitters in vertebrates as there was no identified specific receptor for them (Pei et al., 2016).

The trace amine-associated receptor 1 (TAAR1), a G-protein coupled receptor expressed in these same areas, has been shown to be activated in the brain by TAs, with evidence for this playing a role in the regulation of dopaminergic and psychostimulant action (Grandy, Miller & Li, 2016; Pei et al., 2016). TAAR1 was discovered near-simultaneously by two separate laboratories in 2001 (Borowsky et al., 2001; Bunzow et al., 2001), however research into its role in addiction has not progressed until recently owing to the development of antagonists and agonists that can be used to target this receptor (Lynch et al., 2013; Pei et al., 2016). TAAR1 acts as the mammalian receptor for trace amines in the CNS, having been identified in rodents and primates (Borowsky et al., 2001; Grandy et al., 2016). Due to its responsiveness to TAs and ease of identification in common mammalian model organisms such as rats and mice, TAAR1 is the most researched of the TAAR receptors (Liu & Li, 2018; Pei et al., 2016).

TAAR1 regulation is complex, being activated not only by TAs but also by endogenous transmitters such as dopamine (DA) and serotonin (5-HT), and certain drugs of abuse such as methamphetamine (METH), MDMA, and LSD (Grandy et al., 2016; Pei et al., 2016; Pei et al., 2014). Due to its localisation within the CNS, TAAR1 is unique in that it is able to affect both DA and 5-HT neuronal firing

(Grandy et al., 2016), therefore making it an ideal candidate for designing pharmacological therapies for a range of neuropsychiatric disorders, including substance abuse.

Research into the role of TAAR1 in addictive behaviours has already implicated its role in controlling the addictive effects of methamphetamine and cocaine. Administration of a selective partial TAAR1 agonist reduced cocaine-induced locomotor hyperactivity and cocaine self-administration behaviour in mice (Revel, Mayer et al., 2012). Further studies conducted by Pei et al. (2014) showed that the administration of the partial TAAR1 agonists RO5203548 and RO5256390, prevented context-induced relapse to cocaine seeking after a two-week period of abstinence in rats, as well as drug-induced reinstatement of cocaine seeking. Another study conducted by Cotter et al., (2015) showed that treatment with the partial TAAR1 agonist, RO5203648, prevented the development of METH sensitisation in rats. In self-administration experiments, the partial TAAR1 agonist RO5263397 reduced the break-point for METH in a progressive ratio schedule of reinforcement, suggesting that TAAR1 activation diminishes the motivation to self-administer METH (Pei et al., 2017). Following extinction training in the same rats, administration of the agonist blocked METH-primed reinstatement of METH seeking.

Taken together, these findings showed that TAAR1 activation decreases the stimulant and reinforcing effects of psychostimulant drugs, effectively attenuating self-administration behaviour and relapse in rodent models (Pei et al., 2014; Pei et al., 2016). These effects have also been shown to be selective. Administration of TAAR1 agonists did not have any effect on the self-administration of natural rewards, such as sucrose, in control animals (Cotter et al., 2015; Pei et al., 2014).

These unique effects indicate that TAAR1 may be a modulator of the DA-mediated rewarding and reinforcing effects of addictive drugs (Lynch et al., 2013; Grandy et al., 2016). These reward mechanisms are also known to be involved in producing the same pattern of behaviour in relation to other drugs of abuse, including alcohol (Lynch et al., 2013). In the particular case of alcohol, this may occur through ethanol indirectly altering both DA transmission and TAAR1 signalling due to its ability to affect the levels of endogenous TAAR1 agonists (Lynch et al., 2013). Therefore, this evidence suggests that TAAR1 may be able to regulate alcohol-related behaviours (Lynch et al., 2013).

Evidence suggesting an involvement of TAAR1 in alcohol-mediated behaviours is still scarce. A study conducted by Lynch et al. (2013) was the first to implicate the role of TAAR1 in the reinforcing effects of alcohol via affecting the reward pathways involved in addiction. In this study, both wild-type (WT) and gene knockout (KO) mice (mice who had undergone deletion of the TAAR1 gene) were given ethanol to drink in a two-bottle choice test. The KO mice drank significantly more alcohol than the WT mice. The sedative effects of alcohol, as measured by loss of righting reflex and locomotor tests were also stronger in KO mice, being more heavily affected at lower levels of concentration and for a longer duration, than WT mice. This showed that deletion of the TAAR1 gene in mice results in a stronger preference for ethanol and a greater sensitivity to its sedative effects when compared to controls. These results suggest that modulation of TAAR1 activity may be a novel mechanism to regulate alcohol consumption (Lynch et al., 2013; Grandy et al., 2016).

The Current Study

There are currently no studies on the direct effects of TAAR1 activation with agonists on alcohol-related behaviours, which is the gap in the literature the current study aims to address. The aim of the current study is to therefore examine the potential therapeutic effects of TAAR1 activation on alcohol self-administration and sensitisation behaviours in a rodent model of alcohol addiction. To do this, mice will be trained to self-administer ethanol or sucrose over a period of five weeks using a two-bottle choice paradigm. Specific tests will be conducted to study the effects of TAAR1 activation on alcohol or sucrose consumption and preference. In these tests, mice will be administered with one of three treatments: saline 0.9% (control) or the TAAR1 agonist RO5263397 at either a low (3mg/kg) or high (10 mg/kg) dose. The two-bottle choice test has been widely used in animal studies of alcohol use as a measure of voluntary self-administration and preference for alcohol (Brabant et al., 2014; Lynch et al., 2013).

In addition, the current study will examine the acute locomotor response induced by alcohol and TAAR1's ability to modulate such a response. To this effect, mice will undergo an open field locomotor assay. In this experiment, mice will be injected with one of four treatments (saline + saline [control], TAAR1 low dose + ethanol [low dose], TAAR1 high dose + ethanol [high dose], and saline + ethanol [ethanol]) and placed in an open field apparatus where distance travelled will be measured for 60 minutes. Locomotor behaviours due to drug sensitisation in rodent models are commonly assessed using an open field locomotor tests, with sensitised animals expected to show higher levels of activity on the relevant measurements (Brabant et al., 2014).

Mice have been chosen as the research subjects as animal models have been used extensively in alcohol research due to the resulting pharmacological and behavioural effects being similar to humans (Brabant, Guarnieri & Quertemont, 2014). In the case of mammalian models based on rodents and primates, the underlying structure of the nervous system is also shared with humans, allowing the potential effects and behaviours presenting in humans to be more easily studied in these model systems (Brabant et al., 2014).

The Swiss-Webster strain of mouse was chosen as they have been shown to respond to both alcohol self-administration and locomotor sensitisation challenges (Abrahao et al., 2013; Abrahao, Goeldner & Souza-Formigoni, 2014; Brabant et al., 2014). Furthermore, only males were used due to the majority of studies in this area using male mice only, therefore allowing for systematic comparisons between results.

Hypotheses

Based on findings in previous studies, it is hypothesised that:

1. TAAR1 activation will lead to significantly decreased ethanol intake (g/kg and mL) in a two-bottle choice paradigm.
2. TAAR1 activation will lead to significantly decreased preference for ethanol compared to water (%) in a two-bottle choice paradigm.
3. TAAR1 activation should not significantly change sucrose consumption (mL) and preference (%) in a two-bottle choice paradigm.
4. TAAR1 activation will significantly reduce locomotor activity induced by an acute alcohol challenge in an open-field locomotor assay.

Method

Subjects

38 male Swiss-Webster mice were used for the study. Power analyses for each experiment was conducted using estimations based on the standard deviation (SD) in previous experiments. This used the formula n = 1+2C(s/x) x 2, with *C* depending on significance level (0.05) and power (90%), *s* being the standard deviation of the measured variable, and *x* being the difference that was aimed to be detected in each experiment (National Research Council, 2003). Based on these calculations, and on previous research conducted in this field, a total of 9 units per experimental group (n = 9) was considered appropriate. In the self-administration experiments, the unit of research used was cage, as the mice were pair-housed, resulting in a minimum of 18 mice per experimental group (ethanol or sucrose). For the locomotor study, each mouse was tested singly, thus individual animals were the experimental unit, with a total of 38 animals divided between the four experimental groups (control group [n = 10], low dose group [n = 9], high dose group [n = 10], ethanol group [n = 9]).

Animals were obtained from Australian Bio Resources and acclimated at the Medical Science Precinct (MSP) animal facility one week before the start of the study. Mice were pair-housed in standard mouse cages and were maintained at temperatures of 22°C ± 2, with a 12:12 hour day-night cycle (lights on at 7:00 am). Enrichment was provided in each cage to reduce the effects of impoverished housing, and animals had *ad libitum* access to food. Animals were monitored daily for signs of injury or sickness by the student (monitoring sheets located in Appendix 2 and 3). All housing and procedures were carried out with the ethical approval of the University of Tasmania Animal Ethics Committee (A0018015, Appendix A).

Materials

Self-administration solutions

For the self-administration test solutions of ethanol and sucrose were made by mixing pure (100%) undenatured ethyl alcohol (Sigma Aldrich) and/or white sugar (CSR) with acidified water. Three ethanol solutions at concentrations of 3% ethanol and 3% sucrose, 6% ethanol and 1% sucrose, and 10% ethanol were made for the ethanol group. Three solutions of 3% sucrose, 6% sucrose and 10% sucrose were made for the sucrose group. Solutions were administered via non-spilling water bottles during the training and testing phase.

Open field apparatus

The open-field apparatus was constructed out of Perspex (0.3 x 0.3 x 0.3m). Ethovision XT video tracking software was used to record the distance travelled by mice inside the open field.

Pharmacological treatments

A 20% vv ethanol solution was made by mixing pure (100%) undenatured ethyl alcohol (Sigma Aldrich) with sterile 0.9% sodium chloride (Livingston International). 20% ethanol was injected at a volume calculated using the formula: 2 x (animal weight/160), resulting in a 2g/Kg dose.

TAAR 1 agonist RO5263397 was obtained in powdered form from Hofmann-La Roche Ltd (Switzerland) through a Materials Transfer Agreement and diluted with sterile 0.9% sodium chloride (Livingston International) at concentrations of 0.6 and 2 mg/mL and administered at doses of 3 and 10 mg/kg.

Procedure

Experiment 1: Ethanol and Sucrose Self-Administration

Mice were exposed to a two-bottle choice paradigm in order to assess the effects of TAAR1 on ethanol self-administration. Cages were assigned to one of two groups, one receiving ethanol and the other receiving sucrose, resulting in a total of 10 cages per group. Two mice from the sucrose group were culled due to injuries resulting from fighting before the first self-administration test, reducing the number of mice in the sucrose group to 18 (n = 18). Cages in both groups were equipped with two non-spilling drinking bottles, one containing water and the other containing either an ethanol or sucrose solution depending on their group allocation.

Continuous Training Phase

Mice first underwent training in order to teach them to consume their treatment solutions reliably. During this phase, animals had *ad libitum* access to both one water and one treatment bottle on weekdays, and two water bottles only on weekends for a 3-week period. Bottles were weighed daily by the student to measure consumption (mL), and replenished with fresh solution, before the position of the bottles in the cage being swapped (front or back) to control for side biases. Figure 1 shows a diagrammatic representation of the continuous training phase.

Ethanol Group

For the ethanol group, the concentrations of ethanol in the treatment were gradually increased over the course of three weeks in order to acclimate the animals to the taste and strength of the ethanol. These concentrations were 3%, 6%, and 10%. Each concentration was given over the course of 5 days. To facilitate the drinking of ethanol, a sucrose fading procedure was also implemented for this group. This is a

common and established technique used to initiate ethanol self-administration in two-bottle preference tests in rodent models (Carrillo et al., 2008; Cotter et al., 2015; Samson, Tolliver, Pfeffer, Sadeghi & Haraguchi, 1988; Tolliver, Sadeghi & Samson, 1988). This procedure involved sweetening the ethanol by adding sucrose in order to make it more palatable to the animals, and then fading this out over the course of two weeks. The concentrations used for this were 3% sucrose for the first week and 1% for the second week. This resulted in the final solutions administered to animals in the ethanol group being 3% ethanol and 3% sucrose in week 1, 6% ethanol and 1% sucrose in week 2, and 10% ethanol in week 3.

Sucrose Group

For the sucrose group, the concentrations of sucrose in the treatment were gradually increased over the course of three weeks in order to acclimate the animals to the taste and strength of the sucrose. These concentrations were 3%, 6%, and 10%. Each concentration was offered over the course of 5 days.

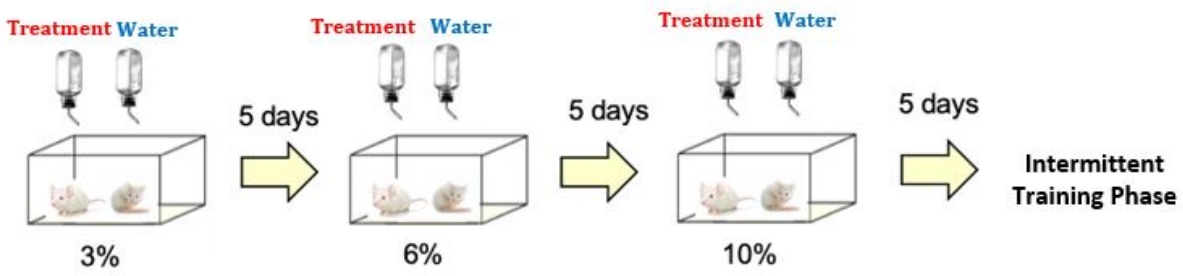

Figure 1. Diagrammatical representation of the continuous training phase

Intermittent training phase

After conclusion of the third week of continuous drinking, animals were then placed on an intermittent exposure schedule for a period of two weeks. This was done to further facilitate drinking of ethanol in the mice (Rosenwasser, Fixaris, Crabbe, Brooks & Ascheid, 2013). Previous studies on alcohol self-administration have

successfully used this method to increase alcohol intake in rodent models (Rossenwasser et al., 2013; Warden et al., 2019). During this phase, animals had access to both one water and one treatment bottle on every second weekday (Mondays, Wednesdays, and Fridays), and access to two water bottles only on remaining weekdays (Tuesdays and Thursdays) and weekends. Please refer to Table 1 for a diagrammatic representation of the intermittent training schedule.

Table 1

Schedule of intermittent exposure. All treatments are at 10% concentration

Week 1	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Bottle 1	Treatment	Water	Water	Water	Treatment	Water	Water
Bottle 2	Water	Water	Treatment	Water	Water	Water	Water
Week 2	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Bottle 1	Treatment	Water	Water	Water	Treatment	Water	Water
Bottle 2	Water	Water	Treatment	Water	Water	Water	Water

Testing Phase

For the testing phase of this experiment, cages from each group (ethanol or sucrose) were randomly assigned to one of three treatment presentation orders using a Latin Square design, as shown in Table 2. The protocol determined the order each animal underwent exposure to the three pharmacological treatments (saline as control, TAAR1 agonist low dose and TAAR1 agonist high dose) in this repeated measures design. The treatments used were 0.9% sodium chloride (control), 3mg/kg of TAAR1 agonist (low dose) and 10mg/kg of TAAR1 agonist (high dose). The agents were administered via intraperitoneal (i.p.) injection, at a volume of 5 mL/kg

of body weight. On the day of testing, animals were deprived of all liquids for four hours in the morning in order to facilitate drinking in the two-bottle choice testing period. After this four-hour period, mice were briefly removed from their home cages to be administered their treatment (saline, TAAR1 agonist low dose or TAAR1 agonist high dose). Mice were then returned to their home cage, which was fitted with two bottles: one containing water and the other containing their treatment (10% sucrose or 10% ethanol). Mice had access to the bottles for four hours. Bottles were weighed before the test, at one-hour intervals during the testing period, and at completion of the testing session to determine the amount of liquid consumed (mL and g/kg of body weight). This was done in order to track drinking patterns over the four hours as the effects of the TAAR1 agonist could begin to wear off after the first hour. After completion of the test session, mice were returned to their intermittent exposure schedule for the rest of the week. This testing procedure was repeated for a total of three times to ensure each cage received all three treatments, with a one-week stabilisation period between each session. Figure 2 shows a diagrammatic representation of this paradigm.

At completion of the final self-administration testing session, animals no longer had access to ethanol or sucrose. During the next 14 days mice had ad libitum access to a single water bottle before undergoing locomotor assessment.

Table 2

Latin-square design for the two-bottle choice paradigm testing order

	Ethanol Group			Sucrose Group		
	Order A N=6 [3 cages]	Order B N=6 [3 cages]	Order C N=6 [3 cages]	Order A N=6 [3 cages]	Order B N=6 [3 cages]	Order C N=6 [3 cages]
Test 1	Saline	TAAR1 High Dose	TAAR1 Low Dose	Saline	TAAR1 High Dose	TAAR1 Low Dose
Test 2	TAAR1 Low Dose	Saline	TAAR1 High Dose	TAAR1 Low Dose	Saline	TAAR1 High Dose
Test 3	TAAR1 High Dose	TAAR1 Low Dose	Saline	TAAR1 High Dose	TAAR1 Low Dose	Saline

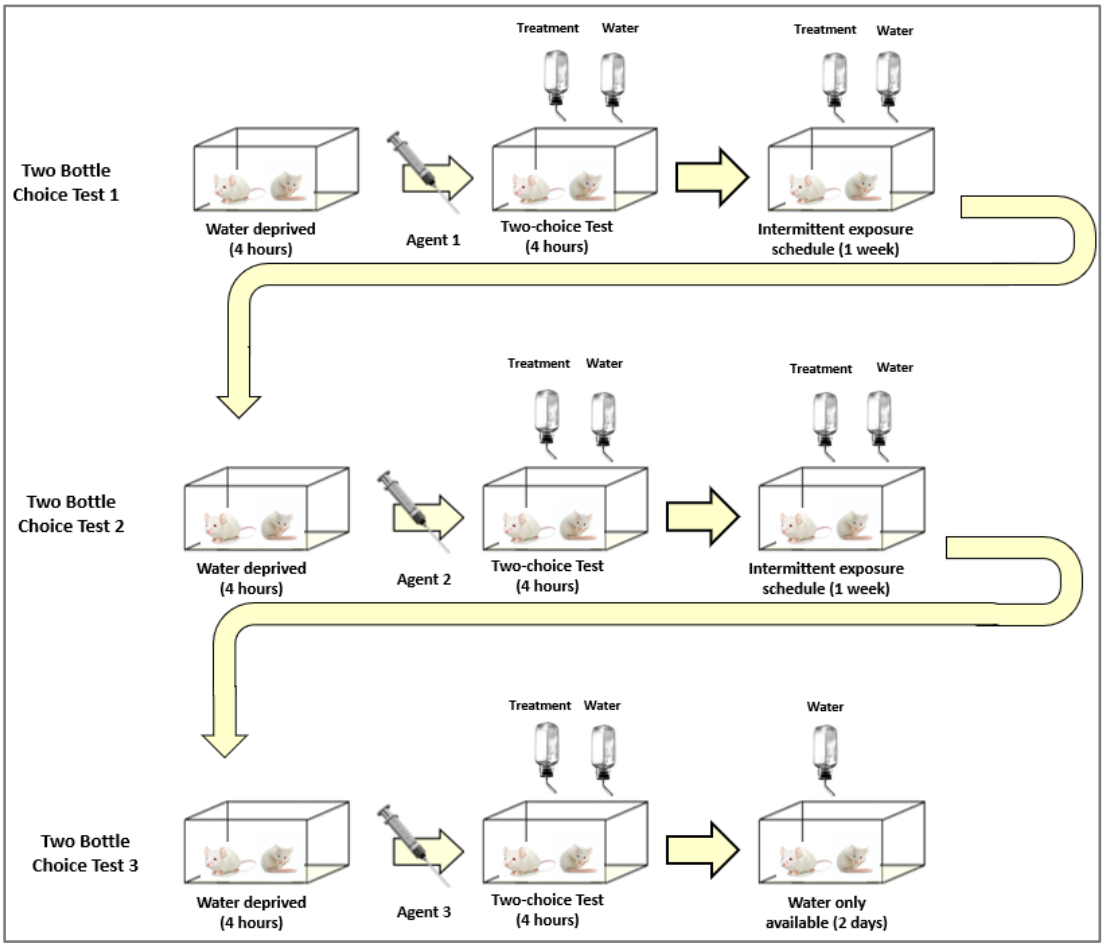

Figure 2. Diagrammatic representation of the two-bottle choice paradigm

Experiment 2: Measuring Ethanol-Induced Locomotor Activity

Mice were semi-randomly assigned to one of four treatment groups: Control (0.9% saline + 0.9% saline), Ethanol (0.9% saline + 20% ethanol), TAAR1 low dose (3 mg/kg of RO5263397 + 20% ethanol) and TAAR1 high dose (10 mg/kg of RO5263397 + 20% ethanol), with group allocation from the previous self-administration test (ethanol or sucrose) counterbalanced across the four treatment groups. There was a total of nine mice per group (n = 9). The mice were injected i.p. with the first agent depending on their group allocation: 0.9% saline (control) and either a low or high dose of the TAAR1 agonist RO5263397. Mice were then placed in the open-field for a 10-minute habituation period, and then injected with the second agent (0.9% saline or 20% ethanol) and placed in the open field for a 60-minute locomotor recording session. Total distance travelled in the hour (cm) was measured. Saline and TAAR1 agonist were injected at a volume of 5ml/kg, and 20% ethanol was injected at a volume calculated using the formula: 2 x (animal weight/160). Figure 3 shows a diagrammatic representation of the locomotor testing procedure. Figure 4 shows how the locomotor fields were arranged and recorded.

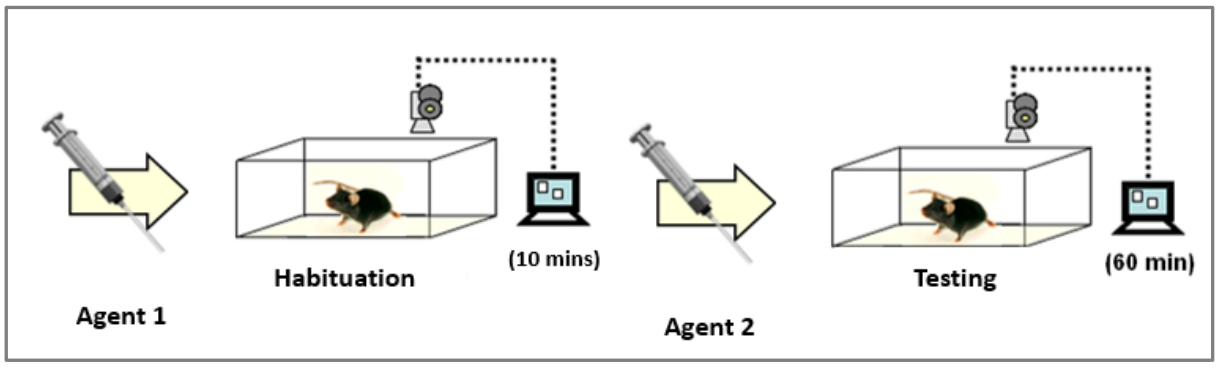

Figure 3. Locomotor assay experimental procedure

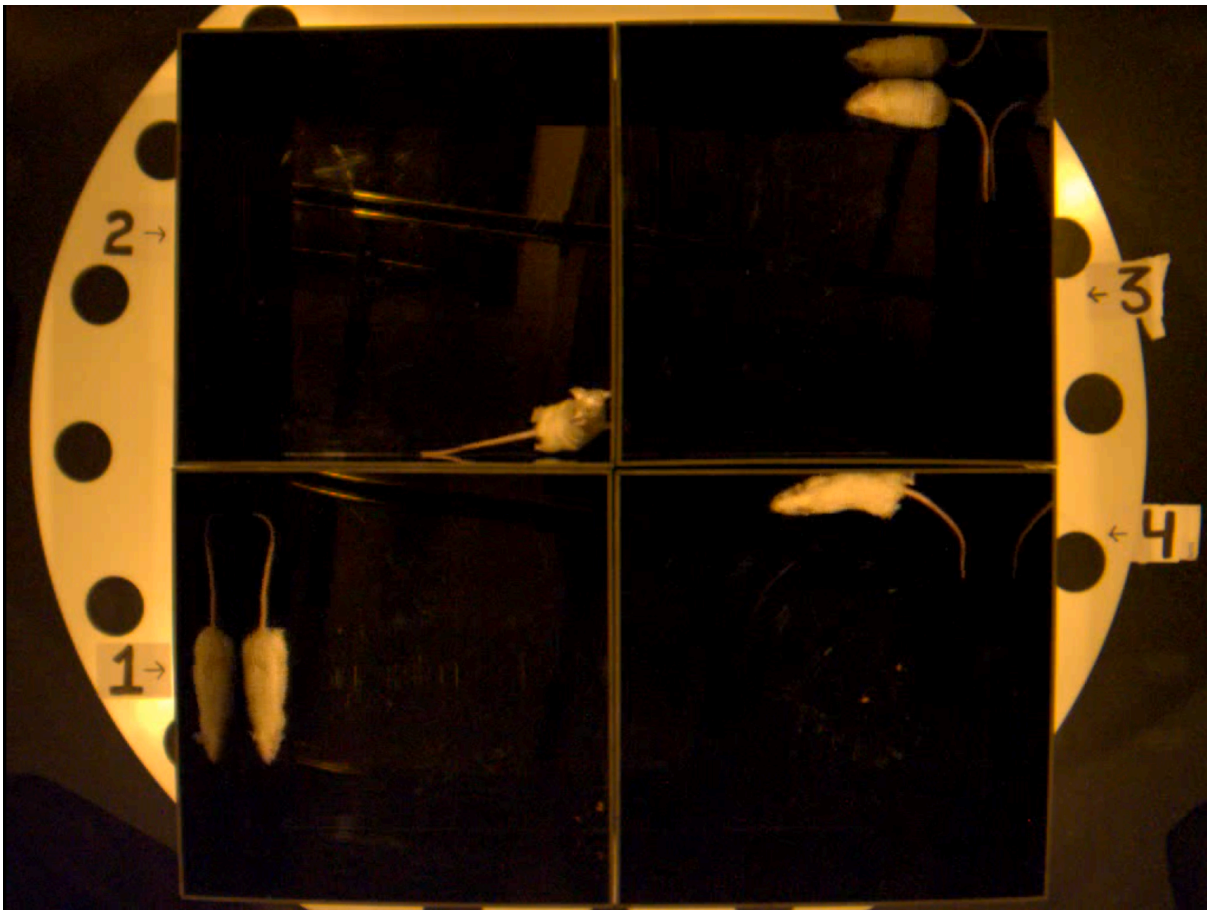

Figure 4. Open field locomotor assay arrangement. Four mice were tracked at a time using Ethovision XT.

Design

The self-administration experiment utilised a counterbalanced within-subjects Latin Square design. Independent variables were treatment (ethanol/sucrose) and dose (saline 0.9%, TAAR1 agonist 3mg/kg, TAAR1 agonist 10 mg/kg). Dependent variables were amount of liquid consumed (g/kg and mL for ethanol; mL only for sucrose) and preference (%).

The locomotor assay experiment utilised a mixed within/between-subjects design. Animals were semi-randomly assigned to treatment groups to ensure even distribution of ethanol- and sucrose-exposed animals across the groups. The independent variable was treatment (control [saline 0.9% + saline 0.9%], ethanol

[saline 0.9% + ethanol 20%], low dose [TAAR1 low dose + ethanol 20%], high dose [TAAR1 high dose + ethanol 20%]). The dependent variable was distance moved in 60 minutes (cm), measured in 10-minute time bins.

Data Analysis

Statistical analyses were conducted in Jamovi (v.1.1.30) and StatView 5.0. Prior to ANOVAs, all data was assessed for normality of distribution, independence of subjects, sphericity, and was also assessed for outliers. For the self-administration paradigm, repeated measures ANOVAs were conducted for consumption and preference of treatment solutions in the continuous training phase, testing weeks and the self-administration test itself. Significant differences and interactions were further assessed using Tukey's post hoc tests, and estimated marginal means using the standard error of the mean were plotted.

For the locomotor assay, a mixed ANOVA with treatment as the between-subjects factor was conducted. Distance travelled over a 60-minute period (cm) was measured in 10-minute time bins for each animal. Two animals were excluded from the locomotor analysis as they exhibited stress reactions prior to, and within the test itself. One-way ANOVAs were conducted to measure distance travelled in the 10-minute habituation period before the test and the total distance travelled in the test itself. Significant effects and interactions were further assessed using Tukey's post hoc tests. Estimated marginal means using the standard error of the mean were plotted.

For all analyses, alpha level was set at 0.05.

Results

Experiment 1: Self-Administration Two-Bottle Choice Paradigm

Training Phase: Continuous Exposure Ethanol Group

Repeated measures ANOVAs showed a significant difference in treatment concentrations for ethanol consumption in both g/kg $F(2, 18) = 6.02$, $p = .010$, $\eta^2_p = .401$, and mL $F(1.18, 10.6) = 69.5$, $p<.001$, $\eta^2_p = .88$ after a Greenhouse-Geisser correction, and ethanol preference $F(1.12, 10.1) = 38.4$, $p<.001$, $\eta^2_p = .81$.

For consumption in g/kg, Tukey's post-hoc tests showed that consumption at 3% was significantly lower than consumption at 6% $p = .008$. For consumption in mL, Tukey's post-hoc tests showed a significant difference $p<.001$, between all three concentrations, with consumption being highest at 3% and decreasing gradually at 6% and 10%, indicating that animals drank less as the concentration of ethanol increased (and the sucrose concentration decreased). For ethanol preference, Tukey's post-hoc tests showed a significant difference between 3% and 10% $p<.001$, and 6% and 10%, $p<.001$. Preference for ethanol solution in comparison to water was highest at 3% and lowest at 10%, indicating that preference for the ethanol solution decreased as the ethanol concentration increased (and the sucrose levels decreased), which was expected.

Training Phase: Continuous Exposure Sucrose Group

Repeated measures ANOVAs found a significant difference between sucrose concentration in terms of sucrose consumed (mL) $F(2, 18) = 4.99$, $p = .019$, $\eta^2_p = .36$, and sucrose preference (%) $F(2, 18) = 16.1$, $p<.001$, $\eta^2_p = .64$.

Tukey's post-hoc tests found a significant difference in terms of sucrose consumed at 3% and 6% $p = .039$, and 3% and 10% $p = .031$, with consumption

showing a gradual increase across concentrations, being lowest at 3% and highest at 10%. Therefore, as sucrose concentration increased, sucrose consumption also increased, which was expected. A similar pattern was found for sucrose preference, with Tukey's post-hoc tests revealing a significant difference between preference at 3% and 6% $p = .007$, and 3% and 10% $p<.001$. As expected, preference for sucrose over water gradually increased over the concentrations, being lowest at 3% and highest at 10%.

Training Phase: Intermittent Exposure Ethanol Group

Repeated measures ANOVAs showed no significant difference between continuous and intermittent exposure in terms of ethanol consumption (in both g/kg and mL) and preference, indicating that intermittent exposure did not have the intended effect of increasing ethanol intake.

Sucrose Group Training Phase: Intermittent Exposure

Repeated measures ANOVAs showed no significant difference between continuous and intermittent exposure in terms of sucrose consumption (mL) and preference. Both sucrose consumption and preference for sucrose increased in the intermittent exposure period, compared to the continuous period, however, as previously shown for ethanol, intermittent exposure did not significantly increase consumption.

Ethanol Group: Two-Bottle Choice Test

A repeated measures ANOVA showed a significant difference between treatment doses $F(2, 18) = 6.09$, $p = .010$, $\eta^2_p = .40$, and a significant interaction

between treatment dose and hour $F(6, 54) = 2.31$, $p = .047$, $\eta^2_p = .204$ in terms of ethanol consumption (g/kg). Tukey's post hoc tests showed a significant difference between saline and TAAR1 agonist low dose $p = .018$, and TAAR1 agonist high dose $p = .021$, with saline-treated animals drinking significantly more ethanol than mice treated with the TAAR1 agonist.

Tukey's post-hoc tests showed a significant difference between saline in the first hour, and TAAR1 agonist low dose in the first hour $p = .019$, second hour $p = .002$, third hour $p = .002$, and TAAR1 agonist high dose in the first hour $p = .005$, second hour $p = .010$, third hour $p = .032$, and fourth hour $p = .005$, saline in the third hour p = .035, and fourth hour $p = .040$. Saline-treated animals drank significantly more ethanol (g/kg) in the first hour than any other group, before decreasing steadily over time. During the second, third and fourth hours, all three groups were drinking ethanol at similar levels (g/kg) (Figure 5).

Repeated measures ANOVAs showed no significant differences or interactions for ethanol consumption (mL) and ethanol preference (%), but did show a significant difference for the factor, hour $F(3, 27) = 4.81$, $p = .008$, $\eta^2_p = .35$, and a significant interaction of dose*hour $F(6, 54) = 3.38$, $p = .007$, $\eta^2_p = .27$ for water consumption (mL). Tukey's post-hoc tests showed saline-treated animals consumed significantly more water in the first hour than in the second $p = .006$ and fourth hours $p<.001$, low dose TAAR1 agonist animals in the second $p = .014$, third $p = .012$, and fourth hours $p = ,014$, and high dose TAAR1 agonist animals in the first $p = .021$ and second hour $p = .003$. Water consumption (mL) for the saline group generally decreased over time, as consumption in the second, third and fourth hours was on a similar level to the agonist-treated groups, which generally drank less (Figure 6).

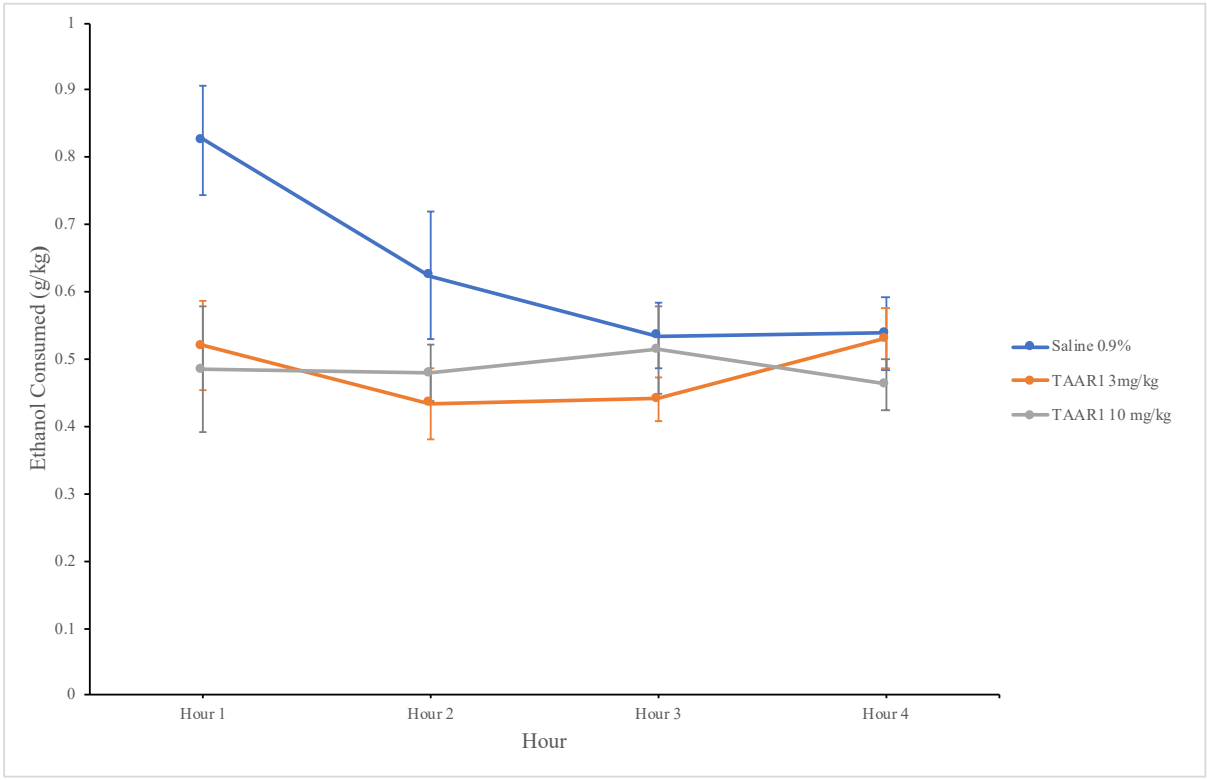

Figure 5. Ethanol consumption (g/kg) in the two-bottle choice test. Error bars are standard errors of the mean (SEM).

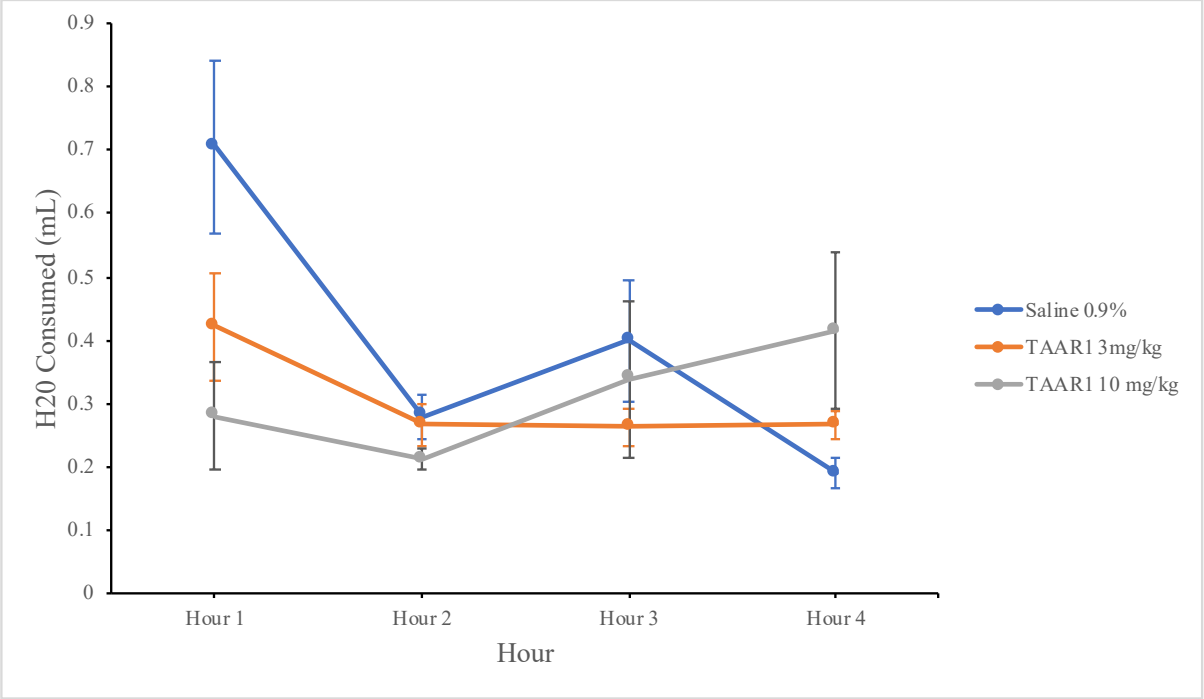

Figure 6. Water consumption (mL) in the ethanol group in a two-bottle choice test. Error bars are SEM.

Sucrose Group: Two-Bottle Choice Test

A repeated measures ANOVA found a significant difference between treatment doses $F(2, 18) = 4.95$, $p = .019$, $\eta^2_p = .35$, and a significant interaction between treatment dose and hour $F(6, 54) = 2,96$, $p = .014$, $\eta^2_p = .25$ in terms of sucrose consumed (mL).

Tukey's post-hoc tests showed a significant difference between saline and the high dose of the TAAR1 agonist $p = .025$, with saline-treated animals drinking significantly more sucrose (mL) than high dose agonist-treated animals in the test overall.

Tukey's post-hoc tests also showed a significant difference between saline in the first hour and second hours $p = .034$, TAAR1 agonist low dose in the first hour $p = .023$, third hour $p = .011$ and fourth hour $p = .035$, and TAAR1 agonist high dose

in the first hour $p = .002$ and second hour $p<.001$. Saline-treated animals drank significantly more sucrose (mL) than mice treated with the TAAR1 agonist in the first hour, before showing a significant decrease in the second hour, with a gradual increase in the last two hours of the test. Saline-treated animals always drank more than animals treated with the TAAR1 agonist across the entire test (Figure 7).

For sucrose preference (%) and water consumption (mL), repeated measures ANOVAs found no significant differences or interactions.

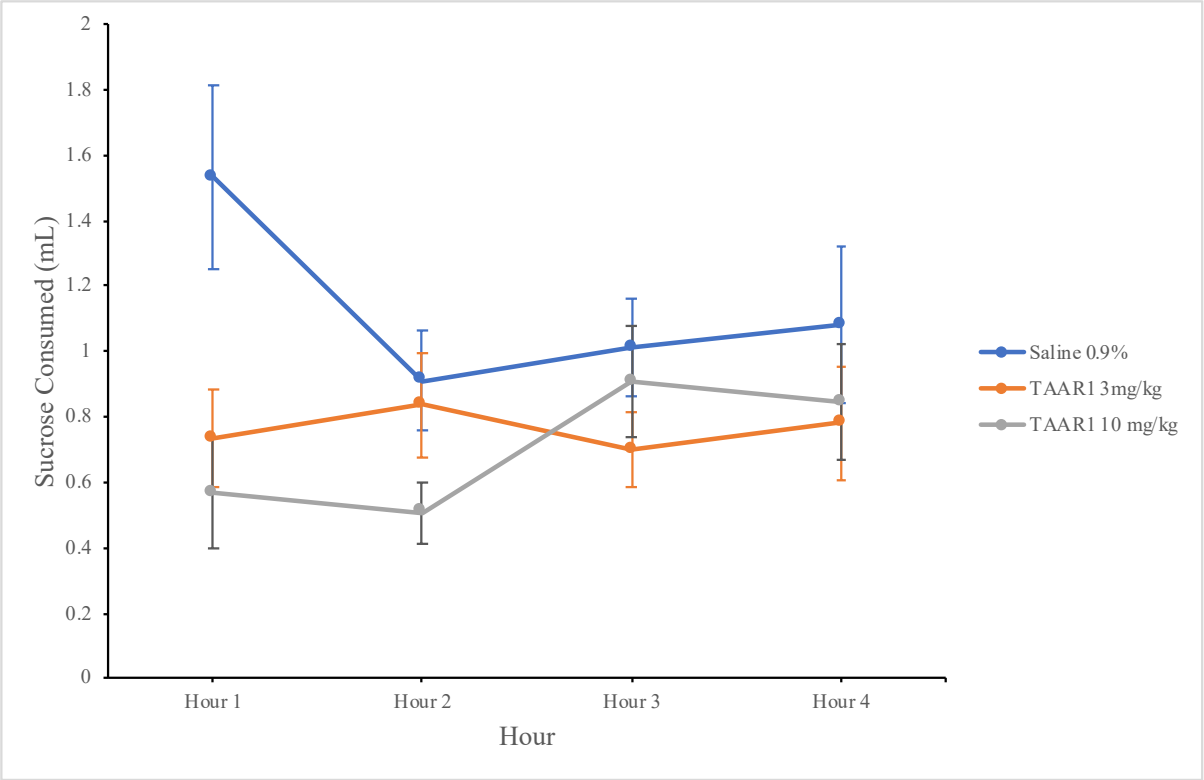

Figure 7. Sucrose consumption (mL) in the two-bottle choice test. Error bars are SEM.

Experiment 2: Open-Field Locomotor Assay

A between-subjects ANOVA found no significant differences between treatment groups in terms of distance travelled (cm) for the 10-minute habituation

period. However, agonist-treated animals showed lower levels of activity than both saline- and ethanol-treated animals.

A mixed ANOVA with treatment as a between-subjects factor showed a significant difference between the time bins $F(2.49, 77.1) = 11.5$, $p<.001$, $\eta^2_p = .27$ after a Greenhouse-Geisser correction, and treatment $F(3, 31) = 7.89$, $p<.001$, $\eta^2 = .25$ in terms of distance travelled (cm). No significant interactions were found.

Tukey's post-hoc comparisons for the factor, time, found a significant difference between the first 10 minutes of the test, and the 10-20 minute bin $p = .007$, 20-30 minute bin $p<.001$, 20-30 minute bin $p<.001$, 30-40 minute bin $p<.001$, 40-50 minute bin $p<.001$, and 50-60 minute bin $p<.001$. This showed that activity for all groups was highest in the first 10 minutes of the test, before decreasing as the test progressed.

Tukey's post-hoc comparisons for treatment found a significant difference between ethanol-treated animals and animals exposed to the TAAR1 agonists $p = .002$, with TAAR1 treatments being able to attenuate ethanol-stimulated locomotor activity across the entire test (Figure 8).

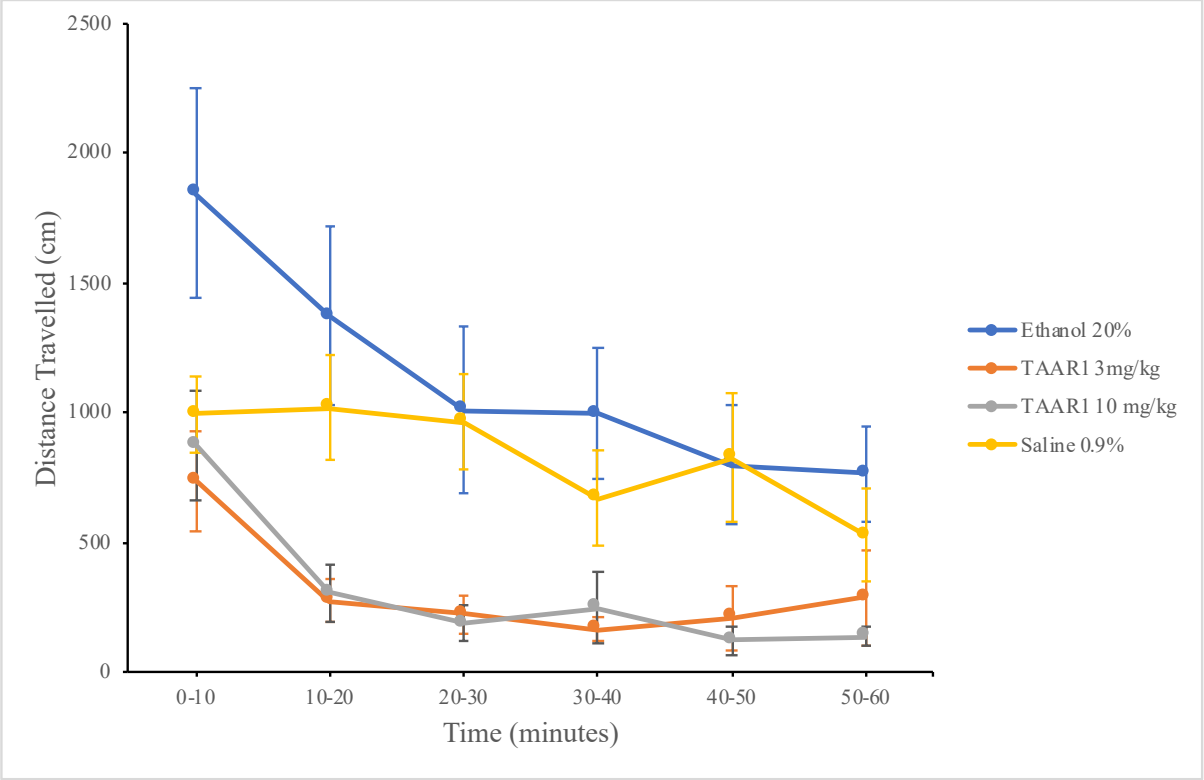

Figure 8. Distance travelled (cm) over 60 minutes in an open-field locomotor assay. Error bars are SEM.

For total distance travelled (cm) in the test, a between-subjects ANOVA found a significant difference between treatments $F(3, 31) = 7.89$, $p<.001$, $\eta^2 = .43$. Tukey's post-hoc tests showed that overall, ethanol-treated animals travelled a significantly greater distance than animals exposed to the TAAR1 agonist $p = .002$ (Figure 9).

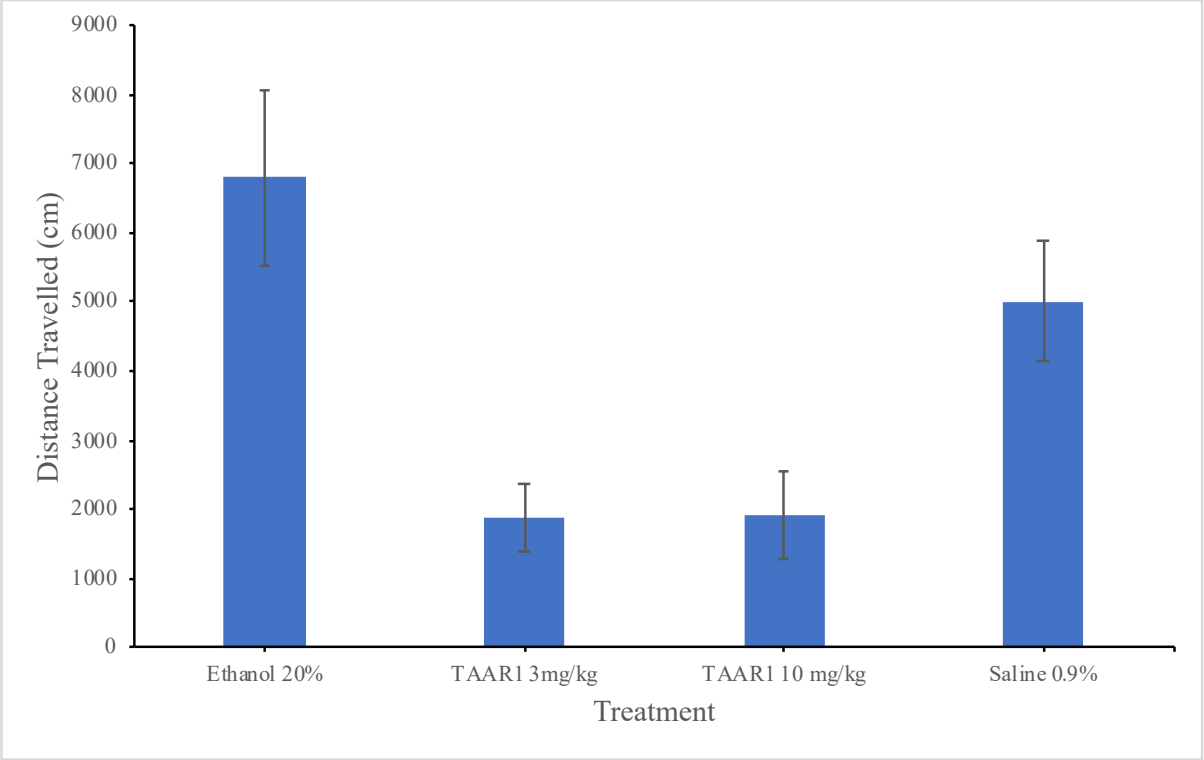

Figure 9. Total distance travelled (cm) in 60 minutes in an open-field locomotor assay

Discussion

The current study aimed to determine the potential therapeutic effects of TAAR1 activation on a range of alcohol addiction behaviours by using a rodent model of chronic alcohol drinking. Animals underwent a two-bottle choice paradigm to assess alcohol self-administration and preference behaviours, and an open-field locomotor assay to evaluate locomotor activity induced by an acute alcohol challenge.

The results of the self-administration experiment showed that, as predicted, administration of the TAAR1 agonist, RO5263397, significantly decreased ethanol consumption in g/kg in the first hour of the preference test. However, RO5263397 also decreased sucrose consumption, and water consumption in the ethanol group,

which was unexpected, suggesting that, at the doses tested, RO5263397 induced non-specific effects. Furthermore, TAAR1 activation appeared to have no effect on ethanol consumption in mL or affect preference for either ethanol or sucrose.

The results of the locomotor sensitization experiment showed that RO5263397 decreased locomotor activity evoked by alcohol treatment, which was expected. There were also no significant differences between the control and ethanol groups except in the first 10 minutes of the test, which was unexpected as ethanol-treated animals were expected to show a significantly higher level of activity than control animals. Furthermore, the significantly reduced locomotion in TAAR1-activated groups compared to both saline and ethanol groups may be due to non-specific effects of the TAAR1 agonist.

Self-Administration Training Phase

The results of the continuous training phase for self-administration were as predicted. Animals in the ethanol group increased their consumption and preference for ethanol when they were transferred from 3% to 6% alcohol, before decreasing at 10%, which was expected due to the removal of sucrose at this concentration. The sucrose group showed a significant increase in consumption and preference for sucrose as concentration increased, which was expected as mice find sucrose solutions highly palatable (Carillo et al., 2008).

Contrary to our prediction, the intermittent exposure period did not significantly increase consumption in either group, with the ethanol group showing a slight decrease at the end of the intermittent training phase in comparison to the end of the continuous training phase, while the sucrose group showed a slight increase. Despite this, animals were still readily consuming their solutions at levels suitable

for conducting the two-bottle preference test. Furthermore, as the animals were drinking for a period of 5 weeks, mice could be said to have engaged in chronic alcohol drinking before the two-bottle preference tests commenced.

Two-Bottle Choice Paradigm

The prediction that RO5263397 administration would significantly reduce alcohol intake in the first hour compared to control treatment was supported in the consumption (g/kg) analysis. Animals treated with the agonist did not increase their general intake over the course of the test, with saline-treated animals significantly decreasing in intake after the first hour to be on par with TAAR1-treated animals.

Interestingly, there also seemed to be no difference in effectiveness between the low and high doses of TAAR1 agonist; both doses significantly reduced drinking in the first hour, with animals treated with the low dose drinking slightly more than those treated with the high dose, though this difference was not significant. This could suggest that the effectiveness of TAAR1 activation on alcohol self-administration behaviour does not depend on dose, which contrasts with studies on METH and cocaine self-administration using self-administration protocols in rats. These studies showed changes in the rate of self-administration was dose-dependent, with higher doses of TAAR1 agonist resulting in lower rates of self-administration (Cotter et al., 2015; Pei et al., 2014; Revel et al., 2012).

Moreover, animals treated with the TAAR1 agonist did not show a rebound increase in consumption at any point, which may imply that TAAR1 activation could be effective in preventing a return to drinking. This could show that treatment with the TAAR1 agonist may reduce the motivation to consume ethanol, which may also support the hypothesis that TAAR1 activation could be beneficial in treating

withdrawal symptoms (Grandy et al., 2016; Lin & Li, 2018; Lynch et al., 2013; Pei et al., 2016). This behaviour was also seen in previous studies conducted on cocaine-addicted animals, where administration of the agonist also prevented a return to drug-seeking in rats (Pei et al., 2014). However, further study on this particular effect is warranted before drawing firm conclusions.

However, the prediction of RO5263397 significantly reducing ethanol consumption was unsupported by the consumption (mL) results. The pattern of consumption was less clear in the mL analysis, which showed no significant effects or interactions after a Greenhouse-Geisser correction, although the effect size for the hour*dose interaction was moderate and similar in size to the g/kg interaction. Animals exposed to the TAAR1 agonist were shown to drink less than saline controls, but this was only a small difference and was not shown to be significant. Furthermore, animals treated with TAAR1 at the lowest dose also showed a large increase in consumption in the final hour of the test, showing a difference between the two doses in terms of effects. This also contrasts with the results of the g/kg analysis.

This could possibly be due to the dose being too low to fully prevent a return to drinking, which was not seen in the high dose animals. Previous studies on cocaine and METH self-administration in rats have shown that higher doses of TAAR1 agonists mitigate relapse to drug-seeking, whereas lower doses are less successful at this (Pei et al., 2014; Pei, Asif-Malik, Hoener & Canales, 2016; Thorn et al., 2014), a finding which appears to be mirrored here. It also makes the conclusion that TAAR1 activation suppresses alcohol withdrawal symptoms less supported at lower doses of the agonist. The seemingly inconsistent pattern with the

g/kg consumption results could also be explained by the amount consumed only increasing slightly by approximately 0.17 mL, which may not be enough to cause a sizeable change in g/kg consumed but was enough to be significantly different in terms of mL consumed. It is also important to note that the g/kg measurements take into account the weight of the animal and therefore provide a more reliable estimate of alcohol consumption and its associated psychopharmacological effects.

The prediction involving ethanol preference was also unsupported by the results. None of the treatment groups were significantly different to each other in terms of preference, with the high dose agonist animals actually showing the most preference for ethanol in the first hour at approximately 48%, compared to the saline and low dose TAAR1 animals which were at approximately 40%. However, the high dose group did decrease in preference over the four hours, becoming the lowest group in the fourth hour while the control and low dose groups gradually increased. Again, this contrasts with previous findings by Lynch et al. (2013) which showed that TAAR1 knockout mice have a significantly higher preference for ethanol in a two-bottle choice test than wild-type, implicating a role of TAAR1 in controlling this variable.

Finally, the prediction that sucrose consumption would be unaffected by TAAR1 activation was unsupported, which is a major difference to previous studies showing that TAAR1 activation has substance-specific effects (Cotter et al., 2015; Pei et al., 2014; Revel et al., 2012). The results of the sucrose consumption (mL) analysis showed that animals treated with the TAAR1 agonist consumed significantly less sucrose (mL) than saline animals in the first hour of the test,

mirroring the results from the ethanol group (g/kg). It appears that the control animals displayed a sharp spike in consumption of sucrose in the first hour of the test, which is not surprising given their liquid deprivation immediately prior to the test commencing. This rate of consumption quickly fell by the second hour after satiation occurred. The significant difference in consumption between the TAAR1 agonist groups (high and low dose) and control group in the first hour is indicative that the TAAR1 agonist is inhibiting the initial spike in consumption that immediately follows liquid deprivation, as seen in the saline group. Therefore, these results show that TAAR1 agonists seem to also have non-specific effects on treatment consumption.

Our prediction in terms of sucrose preference was supported, with no significant differences or interactions found. High dose agonist-treated animals did show less of a preference for sucrose compared to saline and low dose agonist-treated animals, with this increase after the first hour to be approximately the same as the latter two groups by the third hour.

In summary, the results of the two-bottle choice paradigm only supported two of our predictions: administration of a TAAR1 agonist did cause a significant decrease in ethanol consumption (g/kg), and sucrose preference was not affected by administration of the same agonist. RO5263397 did not cause a significant decrease in ethanol consumption (mL) or preference, while producing an unexpected effect by significantly reducing sucrose and water consumption (mL). Therefore, the effects of TAAR1 activation in the current study were not drug-specific.

Based on these results, it is possible that the discrepancies between findings in previous studies and the behavioural results in the present study could be due to the comparatively higher doses of the TAAR1 agonist, as well as species differences (Pei et al., 2017; Thorn et al., 2014). Such dosage, combined with the higher sensitivity of mice to TAAR1 activation, compared to rats, may have reduced motor activity overall in the current experiments (lethargy, impaired motor ability, etc.). This may be supported by the results of the sucrose consumption analysis (mL) and water consumption (mL) analysis in the ethanol group, as both were also shown to be significantly lower in animals exposed to RO5263397 in the first hour. This was unexpected as the effects of TAAR1 activation should be specific to ethanol consumption only. However, if activity was suppressed by the TAAR1 agonist, then this decrease in sucrose and water consumption would also make sense as animals would be less active overall.

Locomotor Sensitisation Test

Our prediction was not fully supported by the results of the open field locomotor activity test. In the first 10 minutes of the test, ethanol-treated animals showed a significantly higher level of activity than the saline and TAAR1-treated animals, which was expected. Furthermore, the activity levels of both saline and agonist animals were approximately the same, seemingly showing that TAAR1 activation blocked the effects of acute ethanol sensitisation on locomotor activity, resulting in a similar activity profile to non-ethanol animals. However, for the remainder of the 60-minute test, ethanol animals decreased in activity to be on par with saline animals. This contrasts with our expectations that acute ethanol-challenged animals would show a significantly higher level of activity compared to

saline controls, therefore exhibiting acute locomotor sensitisation. Therefore, we cannot say that acute locomotor sensitisation occurred in our experiment. Another unexpected result was that agonist-treated animals at both doses showed significantly lower activity than both ethanol- and saline-treated animals. While this did support our prediction that agonist-treated animals would show less activity than ethanol-treated animals, this did not fully support our other prediction that their activity level would be comparable to saline control animals, as it was significantly depressed compared to this group. Interestingly, like the results of the ethanol consumption (g/kg) analysis in the two-bottle choice test, effects on locomotor activity were not dose dependent. Furthermore, these results also seem to support the hypothesis that the non-specific effects of TAAR1 agonist treatment, at least within the dose range used in this study, may suppress locomotor activity overall.

In conclusion, most of our predictions were not fully supported by the results of the current study. While TAAR1 activation did produce a significant reduction in ethanol consumption (g/kg) in a model of chronic alcohol abuse, the same effects were not found for ethanol preference or consumption in mL. Furthermore, these effects were also found in controls, such as water and sucrose consumption, which was unexpected as previous studies have shown that the effects of TAAR1 activation were selective and specific to drugs of abuse (Cotter et al., 2015; Pei et al., 2014; Revel et al., 2012; Thorn et al., 2014).

It is possible that TAAR1 activation through partial agonists can result in other non-specific effects that may cause an overall reduction in activity, which would affect both drinking behaviours and locomotor activity regardless of

treatments or substances consumed (Pei et al., 2017; Thorn et al., 2014). The results of the locomotor test also seemed to support this hypothesis, as activity in agonist-treated animals was significantly depressed and not dose-dependent across the majority of the test when compared to saline-control animals. This was unexpected, as results from previous studies show that agonist-treated animals should display similar levels of activity to control animals, with activity lessening as dose increases (Thorn et al., 2014). Furthermore, as ethanol-treated animals did not show a significantly higher level of activity than saline animals throughout the test, we also cannot conclude that the animals experienced acute locomotor sensitisation.

Possible reasons behind the extent of the non-specific effects observed in this study could be due to the doses used being based on rat studies, which are known not to produce non-specific effects on locomotor activity (Thorn et al., 2014). Despite the similarity between the species, mice have not been used as models in many studies that involve the use of TAAR1 agonists. It is possible that the doses used in the study were either too large for mice, or that mice are possibly more sensitive to the effects of TAAR1 agonists than rats, resulting in an overall depression in activity. Previous studies in rats have reported some mild non-specific effects at high doses of TAAR1 agonists (eg. 10mg/kg), including distractibility and slowness of movement during behavioural tests (Pei et al., 2017; Thorn et al., 2014), which may further support this conclusion. However, evidence in the literature for these non-specific effects is limited, thereby warranting more research in this area.

Regarding the results of the two-bottle choice test, it is also possible the training phase may have been too short to produce a reliable rate of chronic ethanol

intake. Previous studies utilising the same procedure often train mice to consume their treatment at longer intervals and higher strengths until a reliable baseline level of drinking is reached, especially if they are not of an alcohol-preferring strain (Abrahao et al., 2013; Becker & Lopez, 2004; Lynch et al., 2013). For example, Lynch et al. (2013) had mice trained to consume ethanol for 10 weeks, at a higher number of concentrations, which ensured a higher and more reliable rate of chronic drinking. While extending the training phase until a reliable baseline level of consumption had been established was originally planned for this study, this was unable to be implemented due to time constraints. Therefore, it is possible that the mice in the current study simply did not have enough time to become reliably accustomed to drinking ethanol, which may have affected results.

This may also account for the lack of locomotor sensitisation due to acute ethanol challenge in the locomotor test. Previous studies on ethanol-induced behavioural sensitisation in Swiss-Webster mice do show that the development of this effect often depends on individual differences, with individual preference for ethanol being one of the factors that account for the most variability (Abrahao et al., 2013). Non-sensitised animals in this study showed similar levels of locomotor activity to saline control mice after an acute ethanol challenge, which are results mirrored in the current study. Therefore, it is possible that not enough of the animals in the experiment were sensitised to the effects of ethanol prior to the experiment.

In summary, the current study was inconclusive on the possible therapeutic effects of TAAR1 activation in a rodent model of chronic alcohol abuse. While TAAR1 activation did cause a significant reduction in ethanol consumption in g/kg, no effects were found on ethanol consumption in mL or ethanol preference which

contradicted our hypotheses. Furthermore, sucrose and water consumption (mL) was also found to be significantly reduced by TAAR1 activation, which contradicted previous findings and questioned the specificity of the findings. Our hypotheses for locomotor sensitisation due to ethanol were also unsupported as ethanol-treated animals did not show acute locomotor sensitisation behaviours in the majority of the test. While TAAR1-treated animals did show significantly less activity than ethanol animals, they were also significantly lower than control animals, which was unexpected.

Possible follow-ups to this study should consider the use of adjusted doses, such as 0.3 mg/kg and 1 mg/kg, or the use of rats as a model instead of mice, as most research on TAAR1 activation with agonists have been done on rat models. Replications of this study should also consider the use of an extended training phase and assessment of baseline drinking preceding the two-bottle choice paradigm in order to increase the reliability of chronic drinking in the rodent model.

A follow-up to the current study would be especially helpful as this is currently one of very few studies on the effects of TAAR1 on alcohol addiction behaviours and it is the only one thus far to investigate the direct effects of TAAR1 activation with an agonist. Most of the findings of the current study also contradict previous findings in other drug addiction models, therefore further clarification of these results would be especially helpful. There is also a need for more research on the potential therapeutic effects of TAAR1 on a range of alcohol addiction behaviours is especially warranted, as this area of study is still relatively unknown and has a great number of potential benefits for clinicians and patients of an AUD.

The results of this study also warrant further investigation into the possible non-specific effects of TAAR1 activation with agonists in rodent models and

humans, as this may have a multitude of implications for the development of pharmacological treatments that aim to utilise this receptor as a target. More knowledge of these potential effects, especially in humans, is needed before development can progress on these treatments.

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Appendix A: Ethics Approval

UNIVERSITY of TASMANIA

Animal Ethics Committee
ETHICS APPROVAL PERMIT

Office of Research Services
Phone : 03 62267283
Fax: 03 62267148
animal.ethics@utas.edu.au

To: Professor Juan Canales

From: Mel Perry

Date: 23 May 2019

Project: A0018015 - Therapeutic-like effects of TAAR1 activation in models of alcohol abuse

Approved on: 22 May 2019

Approval expires: 21 May 2022

1st Annual Report due: 21 May 2020

Please read this permit carefully as **approval may be withdrawn** for non-compliance with the conditions stated below.

The Animal Ethics Committee has approved the above project and a copy of the initial application document is attached. The approval is subject to the review and AEC approval of an annual report which is due before the approval anniversary. **Please note the due date in your diary.**

As the Responsible Investigator, you MUST ensure that:

1. All aspects of the work conform to the requirements of the current edition of the *Australian code of practice for the care and use of animals for scientific purposes* 8th edition 2013

2. The project is conducted in accordance with the provisions of the Tasmanian Veterinary Surgeons Act 1987 and Veterinary Surgeons Regulations 2012. If the project involves a veterinary service or other animal service, it is **your responsibility** to contact the University Veterinarian to discuss the legal requirements of competency assessment.

3. It is the responsibility of institutions and researchers to be aware of and conduct research in accordance with both general and specific legal requirements, wherever relevant

4. The University Veterinarian and the Animal Ethics Committee are promptly notified of any unexpected event which was not considered in the initial application and impacts on the welfare of any animal directly or indirectly involved in the project.

5. You contact the University Veterinarian to advise when and where your experiments will be conducted. Sufficient notice needs to be given so that an inspection can be easily arranged.

6. In the event of any unexpected death, you contact the University Veterinarian to perform an autopsy.

7. A full record is maintained of all animals used in this project. If at any stage you anticipate the need to use additional animals this must be communicated to the committee before use. Using additional animals without AEC approval is a breach of your ethics permit.

8. That all investigators complete the MyLo Animal Ethics Online Training module every three years.

The project is approved for a maximum of 3 years. If the project is to continue past the expiry date, a new initial application will need to be submitted.

If the investigation necessitates a Parks & Wildlife permit or other permits, you are required to send copies to animal.ethics@utas.edu.au before commencing work.

Executive Officer
Animal Ethics Committee

University of Tasmania **Animal Ethics Committee**	
Ethics Number:	A0018015
Project Name:	Therapeutic-like effects of TAAR1 activation in models of alcohol abuse
Chief Investigator:	Professor Juan Canales
School:	Psychology
Person responsible for day-to-day care:	
Ethics start date:	22 May 2019
Ethics approved to:	21 May 2022
Emergency Contact:	

Appendix B: Self-Administration Monitoring Sheet

Oral Ethanol/Sucrose Self-administration Monitoring Sheet

Animal ID:	**Species/Strain:** Mouse/Swiss Webster	Responsible Investigator: **Juan Canales**	
Earl Clip:	**Sex:** Male	Phone: **0409346027**	
Cage #:	**AEC application No.:** A0018015	Email: **juan.canales@utas.edu.au**	

Day																							
Date																							
# of days at each concentration																							
Bottle Contents																							
Bottle 1 (front)																							
Bottle 2 (back)																							
MONITORING																							
	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal	
Activity																							
Posture																							
Movement/Gait																							
Coat condition																							
Eating/drinking																							
Breathing																							
Alertness																							
Dehydration																							
Eyes/Nose																							
Faeces/urine																							
Monitored by:																							
Action Taken/Other Comments																							

Tick the appropriate box (normal/abnormal). If abnormal is ticked for any clinical observations use the attached scale to give a rating to each abnormal observation, then follow the appropriate intervention for the criteria score obtained.

Appendix C: IP Injection Monitoring Sheet

IP Injection Monitoring Sheet

Animal ID:	**Species/Strain:** Mouse/Swiss Webster	Responsible Investigator: **Juan Canales**	
Earl Clip:	**Sex:** Male	Phone: **0409346027**	
Cage #:	**AEC application No.:** A0018015	Email: **juan.canales@utas.edu.au**	

INJECTIONS																					
Day																					
Date																					
Injection Time																					
Drug/substance																					
Route of injection																					
Volume/dose																					
Animal Weight																					
MONITORING																					
	1Hr Post Inj		**4Hr Post Inj**		**1Hr Post Inj**		**4Hr Post Inj**		**1Hr Post Inj**		**4Hr Post Inj**		**1Hr Post Inj**		**4Hr Post Inj**						
Clinical Observations	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal					
Activity																					
Posture																					
Movement/Gait																					
Coat condition																					
Eating/drinking																					
Breathing																					
Alertness																					
Dehydration																					
Eyes/Nose																					
Faeces/Urine																					
Monitored by:																					
Action Taken/Comments																					

Tick the appropriate box (normal/abnormal). If abnormal is ticked for any clinical observations use the attached scale to give a rating to each abnormal observation, then follow the appropriate intervention for the criteria score obtained.

Appendix D: Clinical Observation Scale for Daily Monitoring

Clinical Observations Scale

This scale is to be used in conjunction with the attached monitoring sheet.

Clinical Observations	**Rating Scale**
UNDISTURBED	
Activity	(*Normal=0; isolated=1; huddled/inactive=2; moribund/fitting/=3)*
Posture	(*Normal = 0; hunched = 2; trembling=3)*
Movement/Gait	*(Normal=0; slight weakness or incoordination=1; reluctance to move or marked weakness or marked incoordination=2; staggering/limb dragging/paralysis=3)*
Coat condition	*(Normal/groomed=0; rough=1; ruffled/unkempt=2; bleeding or infected wounds or self mutilation=3)*
Eating/drinking	*(normal=0; decreased intake during the 1st 24 hrs day=1; decreased intake more than 1 day=2; decreased intake over 48hrs=3)*
Breathing	*(normal=0; rapid, shallow=1; rapid, abdominal breathing=2; laboured, irregular ,extremities/membranes grey/blue=3)*
ON HANDLING	
Alertness	*(normal=0; dull or depressed=1; little response to handling=2; unconscious or aggressive=3)*
Body weight (gm or kg / Score)	*(normal weight & growth rate=0; reduced growth weight=1; chronic weight loss>15% =2; weight loss = or >20%=3)*
Dehydration	*(none=0; skin less elastic=1; skin tenting=2; skin tenting & sunken eyes=3)*
Eyes, Nose	*(normal=0; wetness or dull eyes=1; discharge/squinty eyes=2; coagulated nasal discharge/matted eyes=3)*
Faeces	*(normal=0; moist but formed=1; loose, soiled peri-anal area or mucoid=2; watery or no faeces for 48hrs or blood=3)*
Urine	*(normal = 0; Increased/decreased = 3)*

Criteria for Intervention

- **Criteria for euthanasia:**
  - Assessment score of 3 for any of the following clinical observations: *Activity, Movement/Gait, Breathing, Alertness, or Body weight loss is equal to or greater than 10%* .
    OR

  - A total score of 6 or higher across all parameters.

- **Criteria for immediate veterinary treatment required:**
  - Assessment score of 2 for any of the following clinical observations: *Activity, Movement/Gait, Breathing, Alertness, or Body weight loss is equal to or greater than 10%.*
    OR
  - A total score of 4 or higher across all parameters.

- **Criteria for increased monitoring to twice daily:**
  - A total score of 1-3 across all parameters.